LifeArc

Mycobacterium tuberculosis Molecular Bacterial Load Assay (TB-MBLA) Instructions for Use

36 sample kit

1. Intended use

The ***Mycobacterium tuberculosis*** **Molecular Bacterial Load Assay** (TB-MBLA) kit is an *in vitro* reverse transcription quantitative PCR (RT-qPCR) assay for monitoring the burden of viable *Mycobacterium tuberculosis* (MTB) in human sputum. The TB-MBLA kit is for research use only (RUO) and is not for use in diagnostic procedures. **No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.**

2. Background information

The bacterium MTB is the main causative agent of tuberculosis (TB). Whilst capable of affecting multiple sites, the most common presentation affects the lungs (pulmonary TB). TB is one of the leading causes of death by an infectious agent; the WHO estimates that in 2018 10 million individuals developed TB, with 1.2 million deaths[1]. Although effective treatment is available, this involves a long course of antibiotics and multidrug resistance remains a global challenge.

Standard methods for detecting and monitoring MTB infection are focused on culture or smear microscopy. Culture has been demonstrated to be the more sensitive of these methods but takes considerable time (up to 6 weeks). Molecular based methods, such as qPCR offer faster time-to-results, though tend to focus on the detection of MTB DNA which is a less reliable marker for the presence of viable MTB. The TB-MBLA kit targets ribosomal RNA (rRNA), a stable RNA molecule, as a marker of viability allowing for more accurate monitoring of MTB infection over time. The relative abundance of rRNA transcripts expressed by each MTB cell also increases the sensitivity of the assay for the target species.

3. Product description

The TB-MBLA kit comprises a duplex RT-qPCR assay and extraction control (EC) which also acts as an exogenous control. The assay is designed to detect 16S rRNA of MTB Complex and the extraction control (EC) in different fluorescence channels. The TB-MBLA is specific for the 16S region as this is well conserved for all species and strains of the MTB Complex which can cause tuberculosis in humans[2]. Increases in rRNA expression has been correlated with bacterial growth, and conversely reduction in rRNA correlates with drops in bacterial counts in the presence of antibiotics[3-5]. This correlation, along with the abundance of rRNA make it a good marker of cell viability. In contrast DNA, a much more stable molecule, has been demonstrated to persist long after cell death[6].

The EC included in the kit is a total RNA preparation. The user dispenses a fixed volume of EC into the lysate prior to extraction. The EC can subsequently function as a control for extraction efficiency, PCR inhibition, and to monitor consistency in longitudinal data.

The TB-MBLA kit, for research use only (RUO), has sufficient RT-qPCR reagents for **200 reactions**, and EC for **36 sputum samples**. Optimal use of the TB-MBLA kit reagents is based around performing **two 96-well plates each containing 18 samples.**

4. Kit Contents

This kit contains components which require different storage temperatures. On receipt store the components as described in **Table 1**.

Table 1: TB-MBLA Kit reagents and storage conditions.

Reagent	Box Number	Quantity	Storage Temperature (°C)	Description
TB-MBLA qPCR Master Mix	1	2	-15, -25	1.2 mL 2x master mix contains all reagents required for PCR at 2x concentration. Master mix does not contain ROX.
TB-MBLA Reverse Transcriptase	1	1	-15, -25	50 µL reverse transcriptase enzyme mix.
TB-MBLA Assay Mix	1	1	-15, -25	250 µL duplex assay mix at 20x concentration. Duplex uses FAM™ (Thermo Fisher) and HEX™ (Thermo Fisher Scientific), both quenched by BHQ™-1 (Biosearch Technologies)
RNase-free Water	1	1	-15, -25	2 mL RNase-free water.
	2	1	-65, -85	
TB-MBLA RNA Standard	2	2	-65, -85	100 µL of RNA standard.
TB-MBLA Extraction Control (EC)	2	2	-65, -85	200 µL Extraction Control.

5. Reagents and equipment supplied by customer

If deviating from recommendations, ensure chosen method is as effective and consistent.

- Suitable PPE.
- RNA extraction method.
  - **Recommended lysis strategy:** Mechanical lysis by tissue homogeniser. This kit was optimised using the Precellys 24* (Bertin Instruments) at 6000 rpm for 40 seconds, with Lysing Matrix B (MP Biomedical cat no. 116911050-CF) preloaded bead homogenisation tubes.
  - **Recommended RNA extraction kit:** PureLink™ RNA Mini Kit (Thermo Fisher Scientific), using a higher volume of 700 µl of lysis buffer containing β-mercaptoethanol.
  - **Recommended DNase:** RNase-free DNase set (Qiagen); on-column DNA removal.
- Pipettes* (adjustable).
- Sterile pipette tips with filters.
- Vortex mixer*.
- Benchtop centrifuge* with rotor for 2 ml reaction tubes.
- Benchtop centrifuge* (with sealed removable buckets for 15mL centrifuge tubes), for speeds of 3000 x g for 30 minutes.
- qPCR system.
  - **Recommended system:** CFX96 Touch or CFX96 Connect * (Bio Rad).
- qPCR plasticware suitable for instrument being used, *see manufacturer's guide.*

* Ensure that instruments are checked and calibrated according to the manufacturer's recommendations.

6. Storage and stability

The components of the TB-MBLA kit should be stored at -20°C or -80°C and are stable until the expiry date stated on the label. See **Table 1** for specific storage conditions for each component in the kit. Unnecessary repeated thawing and freezing should be avoided, as this may impact performance. It is recommended to run full 96-well plates (18 samples) at a time to avoid multiple freeze-thaw cycles. If the RNA standards and the EC are to be used only intermittently (>2 freeze-thaw cycles), aliquots should be prepared and frozen until required.

7. Safety and warnings

- The TB-MBLA kit is intended for **research use only and must not be used in diagnostic procedures.**
- This kit should only be used by personnel who have undergone the appropriate training. Prior to use this product insert should be read carefully.
- When working with chemicals and biological material personal protective equipment should be worn including laboratory coat, disposable gloves, and protective goggles.
- All specimens assessed with the TB-MBLA kit should be considered as potentially infectious and handled accordingly.
- For safety information relating to the TB-MBLA Kit components, please consult the appropriate safety data sheet (SDS). The SDS are available online at www.tb-mbla.org/safety.
- Dispose of all unused kit reagents and specimens in accordance with all local, regional, and federal regulations.
- Good laboratory practices for the prevention of contamination during extraction and qPCR set up should be followed. This includes, but not limited to, use of sterile pipette tips, and appropriate laboratory workflows.

8. Directions for use

8.1. Sample preparation

This kit is optimised for RNA isolated from human sputum samples using the above recommended equipment and reagents (**Section 5**). As TB-MBLA is a monitoring tool, it is important that the sample volume, extraction method and elution volume is consistent for all subsequent samples. The user must ensure that the method of RNA extraction used is compatible with downstream use in RT-qPCR.

The sample preparation used is as follows:

1. 1 mL of sputum preserved in 4 mL of guanidine thiocyanate (GTC) solution (50% (w/v) GTC, 0.1 M Tris HCl pH 7.4, 1% (v/v) β-mercaptoethanol) and stored at -80°C (+/- 10°C).
2. Centrifuge at 3000 x g for 30 mins to pellet the cells, discard supernatant and resuspend the cells in lysis buffer (See 8.3).
3. It is strongly recommended to use a mechanical lysis strategy such as a tissue homogeniser, e.g., Precellys 24 (Bertin Instruments). Spike in 10 µL of the EC into the lysate and vortex to mix (see 8.2).
4. For the RNA extraction, the PureLink™ RNA Mini Kit (Thermo Fisher Scientific) with the RNase-free DNase set (Qiagen) are recommended, using a higher lysis buffer volume of 700 µL with β-mercaptoethanol. Any other extraction and DNA removal method will require assessment by the user.

8.2. Extraction control

Each TB-MBLA kit is supplied with sufficient EC for **36 sputum samples**. The kit is optimised for processing 18 samples at a time.

- Ensure the EC is thoroughly mixed by vortexing immediately prior to use.
- 10 µL of EC should be dispensed directly into each sample lysate pre-extraction.

8.3. Reaction preparation and performance

The TB-MBLA kit is supplied with sufficient RT-qPCR reagents for **200 reactions.** The kit is optimised for performing two 96-well plates each with 18 samples (triplicate RT-positive, and a single RT-negative reaction per sample).

- The TB-MBLA RT-qPCR assay is a duplex assay RT-qPCR assay which amplifies MTB and EC targets in the **FAM™** and **HEX™** channels, respectively.
- Except for the TB-MBLA Reverse Transcriptase, all RT-qPCR reagents should be thawed on ice and mixed prior to use. The TB-MBLA Reverse Transcriptase should be kept at -20°C until required.
- Preparation of RT-positive and RT-negative reaction mixes are presented in **Tables 2 & 3** with volumes per reaction**. Note:** It is recommended that an RT-negative reaction is included for each sample to monitor DNA removal during nucleic acid isolation.

Table 2: RT-positive reaction mix preparation.

Reagent	Volume (µL)
TB-MBLA qPCR Master Mix	10
TB-MBLA Reverse Transcriptase	0.2
TB-MBLA Assay Mix	1
RNase-free Water	4.8
Template RNA	4*

* To ensure a consistent concentration, 4 µl of each standard is required. This volume is also recommended for sample RNA extracts; however, if different volumes are required a separate reaction mix should be prepared for standards and samples. The volume of water used should be adjusted accordingly.

Table 3: RT-negative reaction mix preparation.

Reagent	Volume (µL)
TB-MBLA qPCR Master Mix	10
TB-MBLA Assay Mix	1
RNase-free Water	5
Template RNA	4*

* If different sample volumes are required the volume of water used should be adjusted accordingly.

- A dilution series of RNA must be prepared to allow the generation of a standard curve for each plate run. An RNA Standard is supplied for the quantification of MTB. RNA Standard should be used to prepare the top point in the dilution series (i.e., S1 at 1 x 10^7). Further dilutions should be prepared through five 10-fold serial dilutions ($1 \times 10^6 - 1 \times 10^2$ copies/reaction) allowing the generation of a six-point standard curve, **Table 4**. **Note:** it is recommended that each point of the standard curve has triplicate RT-positive reactions.

Table 4: Preparation of dilution series for the standard curve and final concentrations per reaction (4 µL of standard).

Standard	Previous standard (µL)	Water (µL)	Final Concentration (copies/reaction)
RNA Standard	-	-	1×10^8
S1	10	90	1×10^7
S2	10	90	1×10^6
S3	10	90	1×10^5
S4	10	90	1×10^4
S5	10	90	1×10^3
S6	10	90	1×10^2

- When assembling reaction mixes and PCR plates good practices for preventing contamination should be followed.
- Thermal cycling parameters are presented in **Table 5**. When initiating thermal cycling ensure data is collected in both **FAM™** and **HEX™** channels.

Table 5: Thermal cycling parameters.

Cycling Step	Temperature (°C)	Time	Number of Cycles	Acquiring
Reverse Transcription	50	30 minutes	1	No
Activation	95	15 minutes	1	No
Amplification	94	15 seconds	40	No
	60	30 seconds		**Yes**

8.4. Data interpretation

The TB-MBLA assay reports **MTB**-specific amplification in the **FAM™** channel, and **EC**-specific amplification in the **HEX™** channel of a qPCR thermal cycler output file. Guidance of data interpretation is presented below:

- **Standard curve:** Amplification is expected at all dilutions within the series. The standard curve generated should report an efficiency within the range 90-110% (efficiency values outside this range may deliver less accurate results and repeating the run would be recommended.)
- **EC retrieval:** Amplification should be observed in the **HEX™** channel indicating retrieval of the EC. As amplification of MTB target competes with the EC signal, EC positivity is not guaranteed in MTB positive samples. The following acceptance criteria are applied:
  I. **MTB positive samples:** EC signal is not essential.
  II. **MTB negative samples:** EC amplification present.

  These criteria are based on the user adding 10 µl of EC per sample lysate, 50 µL elution volume during the extraction, and 4 µL of sample RNA being assessed during RT-qPCR.

9. Quality control

In accordance with LifeArc's ISO 13485-certified Quality Management System, each lot of *Mycobacterium tuberculosis* Molecular Bacterial Load Assay (TB-MBLA) Kit has been tested against predetermined specifications to ensure consistent product quality.

10. Guide to symbols

REF Product number

Read instructions

Storage temperature

Number of tests

LOT Lot number

Expiry date

Manufacturer

11. References

1. World Health Organisation (2019). Global tuberculosis report 2019. World Health Organisation; Geneva, Switzerland: Document WHO/HTM/TB/2019.
2. Frothingham R, Hills HG, Wilson KH. Extensive DNA sequence conservation throughout the Mycobacterium tuberculosis complex. J Clin Microbiol. 1994;32(7):1639-1643. doi:10.1128/JCM.32.7.1639-1643.1994
3. Aellen S, Que YA, Guignard B, Haenni M, Moreillon P. Detection of live and antibiotic-killed bacteria by quantitative real-time PCR of specific fragments of rRNA. Antimicrob Agents Chemother. 2006;50(6):1913-1920. doi:10.1128/AAC.00869-05
4. Honeyborne I, McHugh TD, Phillips PP, et al. Molecular bacterial load assay, a culture-free biomarker for rapid and accurate quantification of sputum Mycobacterium tuberculosis bacillary load during treatment. J Clin Microbiol. 2011;49(11):3905-3911. doi:10.1128/JCM.00547-11
5. Honeyborne I, Mtafya B, Phillips PP, et al. The molecular bacterial load assay replaces solid culture for measuring early bactericidal response to antituberculosis treatment. J Clin Microbiol. 2014;52(8):3064-3067. doi:10.1128/JCM.01128-14
6. Hellyer TJ, Fletcher TW, Bates JH, et al. Strand displacement amplification and the polymerase chain reaction for monitoring response to treatment in patients with pulmonary tuberculosis. J Infect Dis. 1996;173(4):934-941. doi:10.1093/infdis/173.4.934

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Document History

Revision	Section	Details of Changes	Change Control Number	Initials
6	4 and 8	Update information for dyes	CC/2023/62	RG
5	8	Correction of procedure instruction	CC/2023/11	RG
4	5	Correction of reagent name.	CC/2022/152	RG
3	All	Addition of information including that the master mix provided in the kits does not contain ROX.	CC/2022/134	RG
2	All	See change control	CC/2022/53	JA